Chapter 1

Strategies to detect secondary metabolite production of filamentous fungi using biosensors

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Introduction

Filamentous fungi play an essential role in human welfare and the bioeconomy due to their ability to produce a wide range of compounds such as organic acids, enzymes, secondary metabolites, and active pharmaceutical ingredients[1,2]. The potential of fungal natural products is enormous, as, from 1500 compounds isolated between 1993 and 2001, more than half displayed antibacterial, antifungal, or antitumor activity[3,4]. Many fungal species that are used for the biotechnological production of natural compounds belong to the genus Aspergillus[5,6] or Penicillium[7,8], with *P. chrysogenum* being the most relevant member of the latter[9]. *P. chrysogenum* is well-known for the production of secondary metabolites[11], particularly the β-lactam antibiotic penicillin[10], which saved tens of millions of lives and paved the road for the development of other microbial bioprocesses[11]. It was further predicted that *P. chrysogenum* contains a large number of silent gene clusters encoding for many more, so far unknown, compounds that could be beneficial for human kind[12] (Figure 1).

Lately, new gene-editing methods such as CRISPR-Cas9[13–15] as well as metabolic modeling approaches[16,17] improved the genetic engineering of filamentous fungi remarkably, allowing a precise manipulation, deletion or insertion of genetic pathways to improve the expression of secondary metabolites[12,18]. After successful engineering, highly productive fungal strains, so-called cell factories, can be scaled-up in bioprocesses to enable production of the secondary metabolite in a cost-effective manner[19,20]. However, the efficient identification and selection of strains that have improved expression properties after engineering is often a bottleneck during secondary metabolite production, since commonly used high-throughput screening methods like Fluorescence-activated cell sorting (FACS)[21,22] cannot be used for filamentous fungi as they easily form large multicellular aggregates[23]. As a consequence, the analysis of secondary metabolites predominantly relies on advanced methods like mass spectrometry[24,25] and nuclear magnetic resonance techniques[26,27], which makes the detection and selection of new fungal cell factories a costly and laborious endeavor.

In recent years, metabolite biosensors attracted significant attention for the selection and metabolic engineering of microbial cell factories[28,29]. Being inspired by naturally occurring systems, the detection of metabolites by biosensor is based on highly diverse mechanisms involving multiple biomolecules. After binding to the metabolite, the biosensor undergoes a conformational change that results in a measurable output signal which

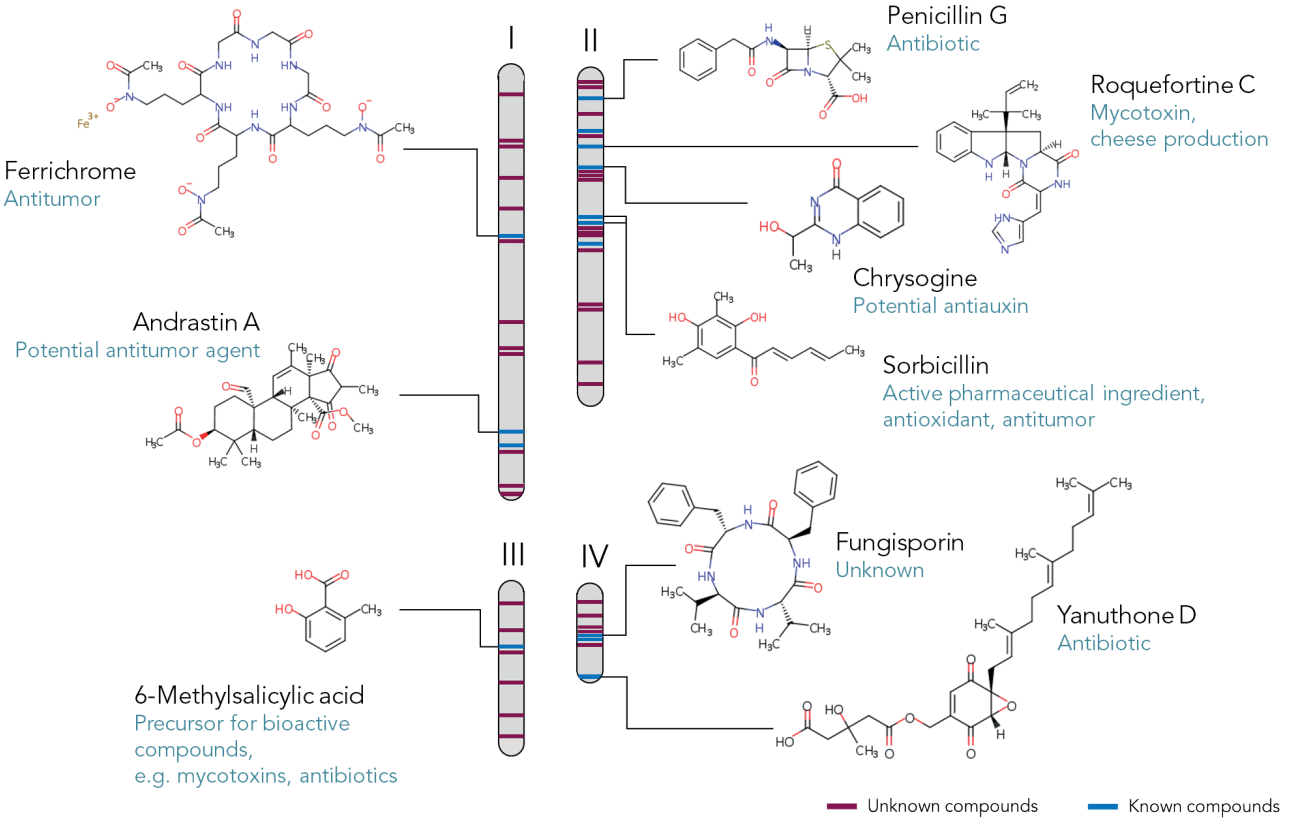

Figure 1 The potential of *P. chrysogenum* to produce valuable compounds is enormous and largely untapped. The locations of known (blue) and predicted (red) gene clusters for the production of secondary metabolites in the four chromosomes of *P. chrysogenum* are depicted (I-IV). Structures of secondary metabolites and their application are shown for a few representative known gene clusters: Ferrichome[33,34], Andrastin A[35,36], 6-Methylsalicylic acid[37], Penicillin G[38], Roquefortine C[39], Chrysogine[40], Sorbicillin[41,42], Fungisporin[43], Yanuthone D[37]. Adapted from [12].

correlates with the metabolite concentration[30]. Even though metabolite biosensors are already applied successfully for the screening and engineering of bacterial[31] and yeast[32] cell factories, the development of biosensors for filamentous fungal cell factories is lagging behind. Given the challenges associated with the high-throughput screening of fungal strains, metabolite biosensors have the potential to improve current screening methods and further expand the metabolic engineering toolbox of fungal cell factories and thereby boost the production of secondary metabolites.

In this chapter, different strategies to detect secondary metabolite production of filamentous fungi using metabolite biosensors will be discussed. We will present a broad range of biosensors that are currently used for the detection of small molecules and will use them as a basis to propose new biosensor designs for the detection of secondary metabolites. Specifically, nucleic acids, proteins, and whole cells will be evaluated as potential biosensors for the detection of penicillin G produced by the filamentous fungus *P. chrysogenum*. Furthermore, advantages and disadvantages of each of these sensor concepts for the development, characterization, and application of penicillin biosensors will be discussed.

Nucleic acid-based small molecule biosensors

Aptamers are single-stranded DNA or RNA oligonucleotides that can fold into specific ligand-binding structures[44–46]. Their ability to bind ligands such as small molecules[47], proteins[48] or cells[49] with high affinity and specificity can be exploited for the development of aptamer-based biosensors[50]. RNA aptamers occur naturally within bacteria, archaea, fungi, and plants, where they regulate protein translation in a ligand-dependent manner as part of so-called riboswitches[51–54]. However, most aptamers used for biosensor development are artificially generated in an *in vitro* selection process called SELEX (Systematic Evolution of Ligands by Exponential enrichment)[44]. In this iterative process, a primary library of up to 10^{15} different DNA or RNA sequences is first incubated with the target ligand. Afterwards, unbound sequences are removed during a washing step, and bound sequences are eluted, amplified and used as new starting library for the following SELEX cycle. After 10-15 rounds of selection, the ligand binding properties of the enriched sequences are evaluated in ligand-binding assays[55] (Figure 2).

Aptamer-based biosensors exist for a multitude of small molecular targets, including toxins[57], food contaminants[58], explosives[59], drugs[60], protein cofactors[61], as well as antibiotics[62]. To demonstrate how aptamers can be applied for the development of small-molecule biosensors, we selected

thee illustrative examples of frequently used sensors. A DNA-, RNA- and RNA riboswitch-based sensor for the detection of small molecules will be presented.

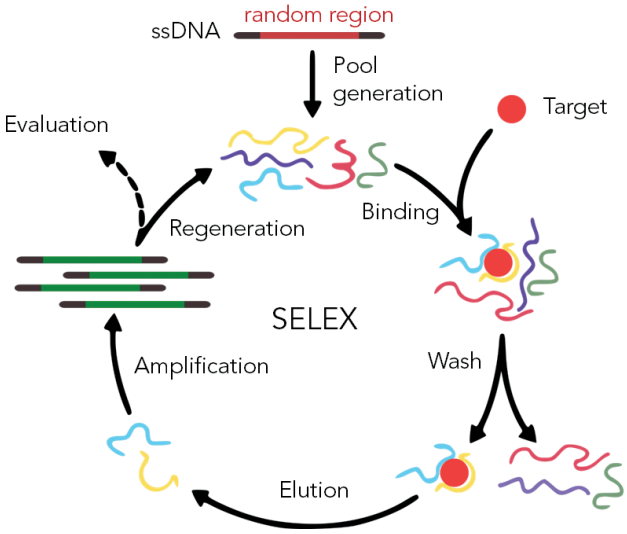

Figure 2 Schematic representation of a classical SELEX cycle to enrich new DNA aptamers for a small molecular target. A pool of up to 10^{15} different ssDNA sequences is incubated with the small molecular target, after which sequences bound to the target a separated from non-binding sequences in a wash step. Binding sequences are then eluted from the target, amplified by PCR, regenerated into ssDNA, and used to start the next SELEX cycle. Sequences are usually evaluated for target binding after several rounds of selection. Adapted from [56]. SELEX: Systematic Evolution of Ligands by Exponential enrichment.

The Ochratoxin A biosensor is an example of a DNA aptamer sensor for the detection of Ochratoxin A[63] (OTA), a mycotoxin produced by different Aspergillus and Penicillium species[64] (Figure 3A). DNA aptamers were enriched during 15 cycles of SELEX whereby OTA was immobilized to the surface of selection beads. After affinity measurements, the aptamer showing the greatest response to OTA was selected for sensor development. The OTA aptamer folds into a specific stem-looped structure, which allows binding of the dye SYBR Green. In the presence of OTA, the stem-loop structure of the aptamer is disrupted, which reduces the amount of SYBR Green that can bind to the aptamer. A linear correlation was found between the signal fluorescence of SYBR Green and OTA, allowing for the detection of OTA in a range of 9 nM to 100 nM[63].

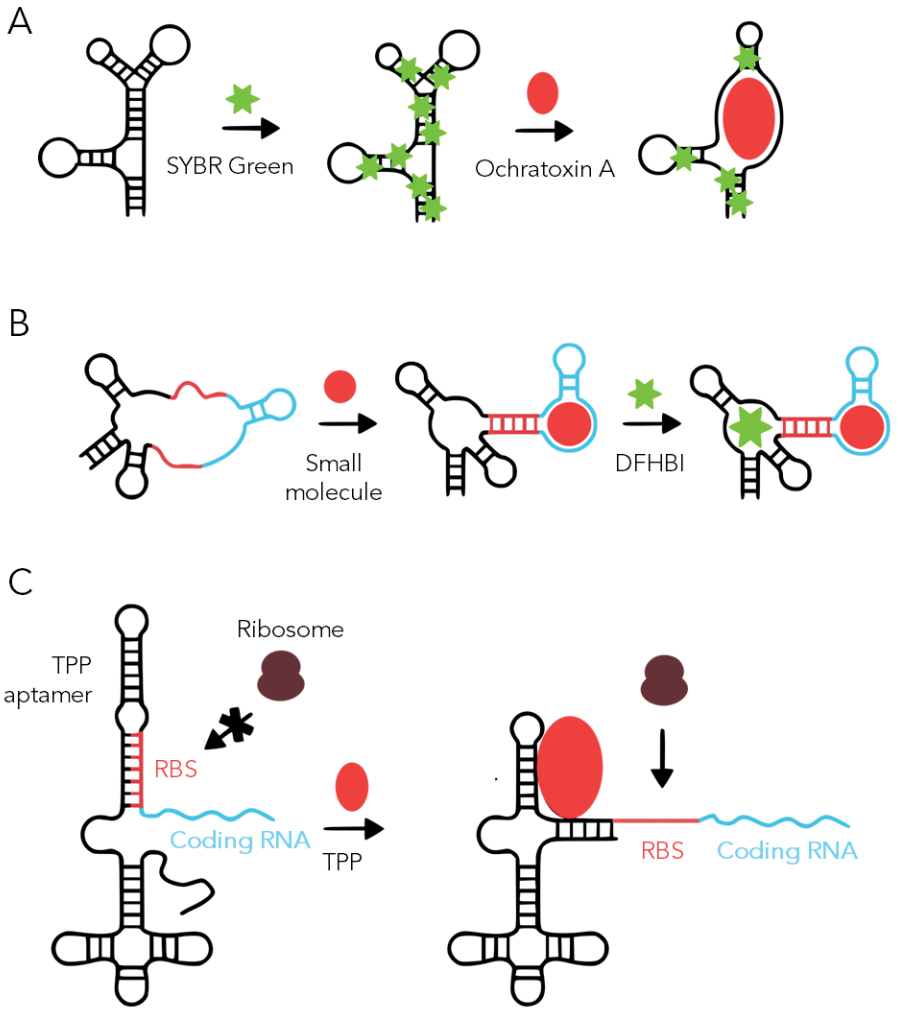

Figure 3 Examples of nucleic acid-based biosensors for the detection of small molecules. A) Ochratoxin A biosensor based on a DNA aptamer. Binding of the fluorescent dye SYBR Green to the aptamer is reduced in the presence of Ochratoxin A, resulting in a decrease in fluorescence[63]. B) Small molecule biosensor based on multiple RNA aptamers. The spinach aptamer (black) is fused to the small molecule aptamer (blue) via a linker sequence (orange). Both aptamers undergo a conformational change upon binding of the small molecule, consequently enabling binding of a fluorophore (DFHBI)[66]. C) Thiamine pyrophosphate (TPP) biosensor based on an RNA riboswitch. In the absence of TPP, the TPP aptamer forms a stem-looped structure with the ribosome binding site (RBS, red), which prevents binding of the ribosome (brown) and prevents translation of a coding RNA sequence (blue) into a detectable protein. Once TPP is present, it binds to the TPP aptamer sequence and induces a conformational change that frees the ribosome binding site and hence enables protein translation[69]. DFHBI: 3,5-difluoro-4-hydroxybenzylidene imadazolinone. Adapted from[70].

A well-known aptamer for the development of RNA-based small molecule biosensor is the so-called Spinach aptamer which can form a fluorescent complex with the fluorophore DFHBI (3,5-difluoro-4-hydroxybenzylidene imidazolinone)[65,66] (Figure 3B). The spinach aptamer was selected after 10 cycles of SELEX whereby DFHBI was immobilized to selection beads. For sensor development, the spinach aptamer is fused to another small molecule-binding RNA aptamer using a linker sequence. Together, both aptamers form a large RNA structure, which is reinforced by binding of a specific ligand to the small molecule-binding RNA aptamer. Only after binding of the ligand, the fluorescent complex between DFHBI and the spinach aptamer can be formed. Spinach-based biosensors were shown to detect a range of small molecules *in vitro* and were used to monitor dynamic changes of intracellular metabolite levels in *E. coli*[66].

In contrast to that, the thiamine pyrophosphate (TPP) riboswitch enables the sensing of intracellular TPP levels by regulating the translation of mRNA gene sequences in *E. coli* (Figure 3C). TPP riboswitches occur naturally in bacteria, archaea, fungi and plants to control expression of thiamine biosynthetic proteins[67]. As the *E. coli* TPP riboswitch was identified first[68], it is one of the most studied riboswitches and was applied for the development of multiple biosensors[69]. In this sensor, the TPP aptamer forms a stem-looped structure with the ribosome binding site, which prevents ribosome binding and translation of an mRNA sequence downstream of the binding site. Binding of TPP to the aptamer induces a conformational change of the riboswitch, freeing the ribosome binding site and enabling translation of an antibiotic-resistant gene. *E. coli* cells containing the sensor can only grow in the presence of the antibiotic in the presence of high TPP concentrations[69].

Strategies for the development of nucleic acid-based penicillin biosensors

From the previous section, it is evident that nucleic acids represent interesting candidates for the development of biosensors targeting small molecules, such as metabolites. They were shown to recognize their target molecule with high specificity and selectivity both, *in vitro* and *in vivo*. With the help of fluorescent dyes such as SYBR Green of DFHBI, an easily detectable signal can be generated and used to quantify metabolite concentrations. Consequently, we see different opportunities for the development of biosensors for the detection of fungal secondary metabolites, such as penicillin. While DNA-based sensors could be applied for the *in vitro* detection of penicillin, e.g. for the development of different bioassays[71], RNA-based sensors could be

integrated into the genome of *P. chrysogenum* to monitor penicillin production *in vivo*, and hence can be used to select for improved production strains.

A recently published penicillin G DNA aptamer[72] could be used for the construction of a fluorescent biosensor, similar to the one developed for the detection of the fungal metabolite Ochratoxin A (Figure 3A). For this, several penicillin G aptamers should be evaluated regarding their ability to bind SYBR Green in the absence and presence of penicillin G and subsequently assessed regarding their ability to monitor penicillin G in different environments, such as cultures of *P. chrysogenum*. Since no RNA-aptamer was published for penicillin to date, the development of an intracellular penicillin biosensor would require the selection of a penicillin RNA aptamer using SELEX. After aptamer selection, Spinach-fusion aptamers or riboswitches like the TPP riboswitch could be designed and characterized regarding their ability to monitor penicillin production in *P. chrysogenum*.

However, the development, characterization, and application of the proposed DNA and RNA penicillin sensors could pose particular difficulties. To construct a DNA-based SYBR Green sensor, dissociation constants of the aptamer-penicillin interaction should be reevaluated to assess whether the aptamers exhibit the desired specificity for penicillin in different environments, such as fungal cultures. Furthermore, chemical modifications of the penicillin aptamers might be necessary to ensure the aptamers are sufficiently stable to sense penicillin fungal culture samples[73]. Since the selection of aptamers targeting small molecules is often accompanied by some particular technical problems, and it has been estimated that less than 30% of selections result in an aptamer[47,74], the generation of new RNA aptamers could represent the main bottleneck during the development of intracellular nucleic acid-based penicillin biosensors.

Protein-based small molecule biosensors

In contrast to nucleic acid-based sensors, practically all protein sensors described in the literature are derived from naturally occurring proteins and are not generated *in vitro* as most aptamers. To function as a biosensor, a protein must exhibit allosteric ligand-binding properties, meaning that ligand binding induces a conformational change of the protein structure, which can be transferred into a detectable readout signal. As a consequence, protein-based biosensors are frequently based on allosteric transcription factors[32,75], allosteric membrane proteins, such as periplasmic binding proteins[76–78], or fusion proteins consisting of at least one allosteric protein[79–81]. Amongst others, ligand-binding can be coupled to a detectable readout signal by generating

fluorescent fusion proteins or via site-specific cysteine labeling with fluorescent dyes[82]. At this, the positioning of the fluorescent label within the protein structure is critical to avoid folding issues and allow ligand-detection using Förster resonance energy transfer (FRET)[83]. FRET sensors can detect changes in the energy transfer between a donor and a receptor fluorescent protein and are frequently used for the development of protein-based biosensors as small changes in distance are sufficient to generate detectable changes in FRET signal[84]. Alternatively, ligand-binding of the allosteric protein can be linked to the catalytic activity of an enzyme by creating protein-enzyme fusion proteins. We selected three examples to illustrate how proteins can be converted into small molecule biosensors.

In our first example, a yellow and cyan fluorescent protein was attached to a glutamine periplasmic binding protein to detect millimolar concentrations of glutamine using FRET (Figure 4A). Binding of glutamine causes a contraction of the protein structure, which moves both fluorescent proteins closer together, resulting in an increased FRET signal. The glutamine sensors was successfully applied *in vivo* to monitor glutamine concentrations for up to eight days of cell culture[85].

Another method to visualize protein-small molecule interactions is based on bimolecular fluorescence complementation[86,87]. A protein sensor for estrogenic compounds was built by taking advantage of two complementary fragments of a fluorescent protein, which solely form a functional protein complex when brought into proximity (Figure 4B)[88]. Two complementary parts of a yellow fluorescent protein were fused to an allosteric estrogen receptor protein, which adapts a closed confirmation in the presence of estrogenic compounds, enabling complementation of the yellow fluorescent protein. Multiple different agonistic and antagonistic estrogenic compounds could be discriminated using this BiFC-based biosensor[88].

In our last example, a fusion protein was created of a maltose-binding protein (MBP) and a β-lactamase enzyme for the detection of maltose in *E. coli* cells (Figure 4C)[89]. In the absence of maltose, the MBP adapts an open structure, thereby rendering the catalytic site of the enzyme inactive. Once maltose binds to the MBP, it changes from an open to a closed form, which restores the catalytic activity of the β-lactamase. Hence, cells expressing the fusion protein can grow on β-lactam antibiotics in the presence of maltose as signal output[89].

Strategies for the development of protein-based penicillin biosensors

Protein-based sensors are useful tools for the *in vivo* detection of small molecules. However, the number of known proteins that undergo a conformational

change in the presence of the desired target molecule determines the number of potential protein-based sensors and hence represents a major bottleneck in sensor development. To assess the potential of proteins for the development of a penicillin biosensor, we identified a range of proteins interacting with penicillin, which are either involved in the production of penicillin, affected by penicillin or causing penicillin resistance.

A critical protein involved in the production of penicillin G, is the enzyme isopenicillin N acyltransferase (IAT), which catalyzes the final step during penicillin G synthesis in *P. chrysogenum*[90]. In its active form, IAT consists of two protein subunits, which were shown to undergo a conformational change during binding of the penicillin G precursor molecule 6-aminopenicillanic acid (6-APA)[91–93]. Therefore, the design of fluorescent IAT fusion proteins could allow to link catalytic activity of IAT to changes in FRET intensity and consequently allow biosensing of penicillin production in *P. chrysogenum*.

Another class of protein that is well-known for its interactions with penicillin is the bacterial penicillin-binding proteins (PBPs)[94]. Since PBPs are essential for cell wall synthesis in many bacteria, binding of penicillin to PBPs is directly linked to cell death[38]. Since PBPs were not shown to undergo a conformational change upon binding of penicillin[94], the development of PBP-based biosensors would require engineering of the PBP protein structure to ensure that penicillin binding induces a conformational change which can be linked to a fluorescent signal or the catalytic activity of an enzyme fused to PBP. The presented maltose biosensor (Figure 4C) could serve as a blueprint for such a sensor, even though an enzyme without catalytic activity for β-lactams should be chosen in this case.

We further identified two bacterial proteins involved in penicillin resistance, that could be used for biosensor development, namely β-lactamases, which are secreted by different bacteria to degrade β-lactam antibiotics[95] and the transcription factor TcaR which is expressed by different *Staphylococcus* species to induce biofilm formation in the presence of penicillin[96,97]. To link the activity of a β-lactamase to a detectable readout, engineering of the β-lactamase protein structure would be required to generate mutant proteins exhibiting the desired allosteric properties, e.g. by directed evolution[98]. In the case of TcaR, different fluorescent biosensor designs are conceivable, since crystal structures revealed that TcaR undergoes a conformational change upon binding to penicillin to dissociate from its promoter DNA[97].

Even though we could identify several proteins that directly interact with penicillin, the development of penicillin biosensors based on those proteins could face some severe challenges. In the case of the IAT and the TcaR protein, the generation of fluorescent fusion proteins could interfere with protein folding[83]

as frequently used fluorescent proteins, such as GFP, YFP, and CFP, are relatively large (~ 27 kDa[99]) compared to the IAT (11 and 29 kDa[93]) and TcaR subunits (both 17 kDa[97]). Instead of creating fluorescent fusion proteins, cysteines within the protein structure could be labeled with fluorophores enabling FRET detection[82].

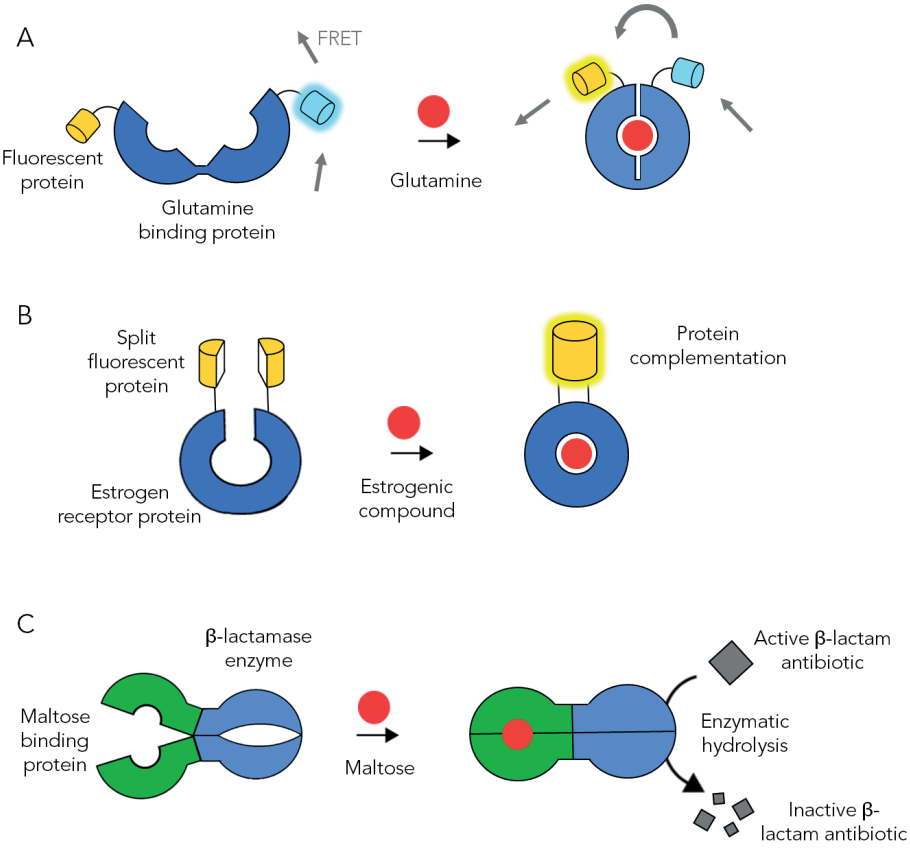

Figure 4 Examples of allosteric protein-based biosensors for the detection of small molecules. A) FRET-based glutamine sensor. Binding of glutamine induces a conformational change of a glutamine binding protein equipped with a yellow and a cyan fluorescent protein, which moves both fluorophores into closer proximity, resulting in an increased FRET[85]. B) Sensor for estrogenic compounds based on fluorescence complementation. Two complementary fragments of a yellow fluorescent protein were fused to an estrogen receptor protein. In the presence of estrogenic compounds, the protein adopts a closed structure, resulting in the complementation of the fluorescent protein[88]. C) Maltose biosensor based on the fusion of a maltose-binding protein (MBP, green) and a β-lactamase enzyme (blue). In the absence of maltose, the structural conformation of the MBP disturbs the structure of the enzyme, rendering it inactive. When maltose is presence, the enzyme's structure is restored which results in the enzymatic hydrolysis of β-lactam antibiotics, allowing cells expressing this biosensor to grow in the presence of β-lactam antibiotics[89]. Adapted from[70]. FRET: Förster resonance energy transfer.

While TcaR would need to be engineered to contain cysteines for labeling, IAT contains cysteines in both protein subunits[93,97]. In the case of PBPs and β-lactamases, the development of penicillin biosensors would require random mutagenesis of both protein structures individually or different fusion proteins, followed by high-throughput screening to identify mutants exhibiting the desired ligand-induced switching properties[9870]. Even though protein-based biosensors were successfully selected using similar strategies, such as the shown maltose biosenor[89], the generation of mutant libraries and the subsequent screening for improved functions are laborious and complex processes.

Whole-cell and cell-free small molecule biosensors

Many nucleic acid- and protein-based small molecule sensors can be engineered to function inside cells or cell-free systems. In both cases, the transcription and translation machinery of the cell or the cell-free system is used to generate a fluorescent signal in a metabolite dependent manner. In this section, we will first present whole-cells and subsequently cell-free system for the development of small molecule biosensors.

Whole-cell biosensors are commonly applied for selection of single, high metabolite-producing cells. For instance, a range of riboswitches were engineered to enable selection of new cell factory strains, with the Spinach-TPP[100] and the so-called suicide riboswitch[101] being prominent examples. Besides riboswitches, transcription factors are frequently used for the development of genetically encoded whole-cell metabolite biosensors[102]. In one example, a whole-cell *E. coli* sensor was developed, where β-lactam antibiotics trigger a series of pathway reactions, which eventually result in expression of GFP (Figure 5A)[103]. Hereby, metabolite detection is based on the transcriptional repressor AmpR, which turns into a transcriptional activator in the presence of a cell wall degradation product formed upon β-lactam uptake. The whole cell sensor was shown to detect a broad range of β-lactam antibiotics including amoxicillin, ampicillin and penicillin G in the low nanomolar to high picomolar range, well below concentrations that are cytotoxic to bacteria[103].

In another example, the prokaryotic transcriptional activator BenM was engineered as a *cis,cis*-muconic acid (CCM) sensor in the yeast *Saccharomyces cerevisiae* (Figure 5B)[104]. After optimization of the sensor design by high-throughput engineering and screening of BenM variants with improved CCM inducibility, the sensor enabled high-throughput *in vivo* selection of high producing yeast cells. Up to 1.4 mM CCM could be detected using the BenM sensor, rendering it a suitable candidate for screening high-producing CMM strains during early stages of cultivation[103].

Two-component regulatory systems (TCSs) are another major source for the development of whole-cell biosensors[105]. TCSs are most common in bacteria and typically consist of a membrane-bound histidine kinase that senses the input stimulus and a response regulator protein that mediates the expression of a target gene[106]. A range of highly sensitive TCS-based sensors has been developed for the detection of small molecules in *E. coli* such as aspartate, tetrathionate and thiosulfate[107].

In comparison to whole-cells, biosensors based on cell-free systems are less frequent and are mostly applied for the development of *in vitro* diagnostics. In bacterial cell-free systems, all essential components for transcription and translation are derived from either *E. coli* extract or from purified components, thereby reducing the complex interactions found in a whole cell to the bare minimum. In our example, a cell-free system was combined with a synthetic gene network to detect a tetracycline analog (Figure 5C)[108]. When incubated with the cell-free transcription-translation system, the transcriptional repressor TetR is expressed from the synthetic gene network and binds to the promoter of a GFP-encoding gene, thereby preventing GFP expression. Binding of TetR to the promoter is reversed in the presence of the tetracycline analog, resulting in GFP expression. A great advantage of cell-free sensors, such as the presented TetR-sensor, is that they can be coupled to a colorimetric output for metabolite detection by eye and freeze-dried onto paper, thereby enabling a simple application of the sensor[108].

Strategies for the development of whole cell and cell-free penicillin biosensors

Taken together, whole cell sensors are beneficial for the screening of microbial cell factories, whereas cell-free biosensors are convenient to detect metabolites in liquid samples. While whole sensors are genetically integrated into cells producing the target metabolite, cell-free biosensors require a synthetic gene networking encoding for the sensor and a reporter system, as well as a compatible cell-free system. As both sensor concepts are usually built upon existing nucleic acid- and protein-based biosensors, a new sensor does not only require the availability of a well-studied nucleic acid- or protein-based sensor but further needs to be fine-tuned to function in a cellular or cell-free environment. In the case of whole cell sensors,

synthetic biology and metabolic engineering tools are therefore required to successfully integrate the sensor into the cells. For cell-free systems, sensor expression and metabolite recognition need to be conducted by the cell-free transcription and translation machinery.

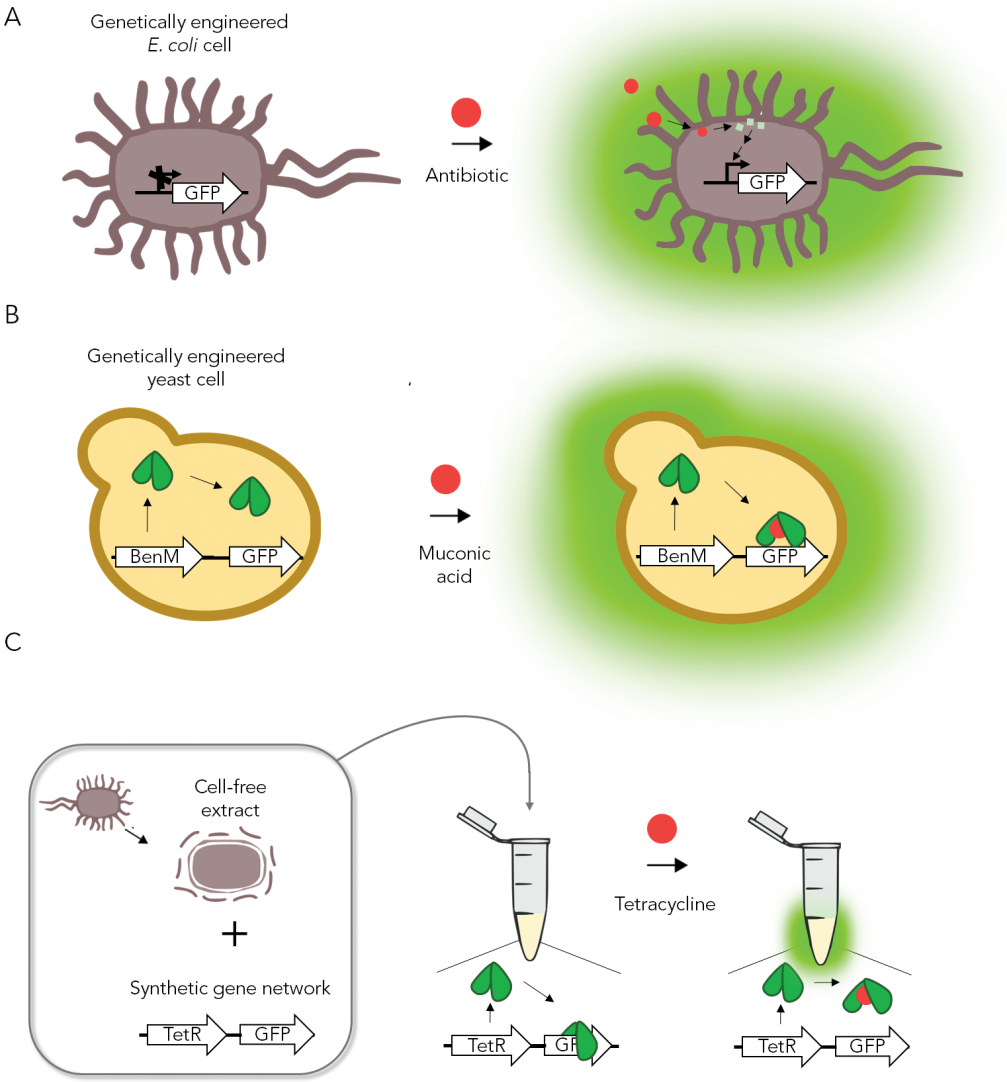

Figure 5 Examples of two whole-cell and one cell-free biosensor for the detection of small molecules. A) *E. coli* was genetically engineered in such a way that cell-wall breakdown byproducts induced by β-lactam antibiotics cause expression of a green fluorescent protein (GFP)[103]. B) The yeast *S. cerevisiae* was genetically engineered to express the transcriptional activator BenM (green), which increases expression of GFP in the presence of cis,cis-muconic acid[104]. C) A synthetic gene network encoding for the TetR repressor and a GFP protein is transcribed and translated in cell-free *E. coli* extract. GFP is only expressed in the presence of a tetracycline analog, which causes dissociation of TetR from the GFP promoter[108].

As a consequence, we currently see three options for the development of a penicillin sensor, namely engineering of bacterial cells for the detection of penicillin in the surrounding of the penicillin producer *P. chrysogenum*, the integration of a penicillin sensor into the genome of *P. chrysogenum*, or the construction of a sensor in a cell-free system for the detection of penicillin in *P. chrysogenum* culture samples.

To monitor penicillin production in the surrounding of *P. chrysogenum*, *E. coli* biosensor cells (Figure 5A) could be co-cultured with *P. chrysogenum* producer strains to enable selection of high penicillin-producers based on GFP expression. One way to enable screening of those co-cultures in high-throughput could be the application of nanoliter reactors, as they were successfully applied to select *B. subtilis* strains with improved vitamin B2 production using co-cultured *E. coli* vitamin sensor strains[109]. Lately, a library of the filamentous fungus *A. niger* was successfully screened for improved enzyme activity using nanoliter droplets[110]. However, further research on the co-cultivation of filamentous fungi and bacteria in nanoliter reactors would be needed to assess the robustness of this screening system. Furthermore, the *E. coli* biosensor operates best at nanomolar concentrations of penicillin[103], making the sensor unsuitable for the screening of high producing *P. chrysogenum* strains reaching penicillin concentrations in the high millimolar range[10].

A genetically encoded penicillin sensor in *P. chrysogenum* could be developed with the transcriptional regulator TcaR from *S. epidermidis*, which was shown to dissociate from DNA in the presence of penicillin[97]. Similar to the BenM sensor in yeast, TcaR could control the expression of a fluorescent protein (Figure 5B), whereas expression is turned off in the absence, and on in the presence of penicillin. Only recently, a synthetic transcription factor was expressed in *P. chrysogenum* to improve expression of secondary metabolites[111], thereby demonstrating that tuned expression of heterologous transcription factors in possible in this fungus. However, additional information on the TcaR-DNA and -penicillin-binding properties would be necessary to rationally engineer a TcaR-based biosensor in *P. chrysogenum*.

Similar to the genetically encoded penicillin sensor, a synthetic gene network could be constructed based on the TcaR system for the detection of penicillin in a cell-free transcription translation system. As for the TetR biosensor (Figure 5C), penicillin would be detected via the expression of a GFP protein which is solely transcribed in the presence of penicillin. As the TcaR regulator and the TcaR promoter are derived from *S. epidermidis,* their feasibility to function in an *E. coli* cell-free system would need to be assessed in the first place.

Conclusions

Given the tremendous chemical diversity and specificity of biomolecules such as nucleic acids, proteins and enzymes for the detection of small molecules, metabolite biosensors have the potential to revolutionize current strategies for cell factory development. Especially for the engineering and screening of filamentous fungal cell factories, biosensors represent an exciting alternative to current screening methods, as they could boost the production of beneficial secondary metabolites.

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